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Ahvenainen, Terhi V.

2018-12-15

Ahvenainen , T V , Mäkinen , N M , von Nandelstadh , P , Vahteristo , M E A , Pasanen , A M , Bützow , R C & Vahteristo , P M 2018 , ' Loss of ATRX/DAXX expression and alternative lengthening of telomeres in uterine leiomyomas ' , Cancer , vol. 124 , no. 24 , pp. 4650-4656 . https://doi.org/10.1002/cncr.31754

http://hdl.handle.net/10138/323867
https://doi.org/10.1002/cncr.31754

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Original Article

Loss of ATRX/DAXX Expression and Alternative Lengthening of Telomeres in Uterine Leiomyomas

Terhi V. Ahvenainen, MSc[1,2]; Netta M. Mäkinen, PhD[1,2]; Pernilla von Nandelstadh, PhD[1,2]; Maija E. A. Vahteristo, BSc[1,2]; Annukka M. Pasanen, MD[3]; Ralf C. Bützow, MD, PhD[3]; and Pia M. Vahteristo, PhD[1,2]

BACKGROUND: Uterine leiomyomas (ULs) are the most common gynecologic tumors and affect 3 of every 4 women by the age of 50 years. The majority of ULs are classified as conventional tumors, whereas 10% represent various histopathological subtypes with features that mimic malignancy. These subtypes include cellular and mitotically active ULs and ULs with bizarre nuclei. Uterine leiomyosarcoma (ULMS), the malignant counterpart of UL, is an aggressive cancer with poor overall survival. The early diagnosis and preoperative differentiation of ULMS from UL are often challenging because their symptoms and morphology resemble one another. Recent studies have shown frequent loss of alpha-thalassemia/mental retardation syndrome X-linked (ATRX) or death domain–associated protein (DAXX) expression in ULMS, and this is often associated with an alternative lengthening of telomeres (ALT) phenotype. **METHODS:** To investigate ATRX and DAXX expression and the presence of ALT in UL subtypes, immunohistochemical and telomere-specific fluorescence in situ hybridization analyses were performed. The study material consisted of 142 formalin-fixed, paraffin-embedded tissue samples representing various UL subtypes and 64 conventional ULs. **RESULTS:** A loss of ATRX or DAXX and/or ALT was detected in 6.3% of the histopathological UL subtype samples (9 of 142). Two patients whose ULs showed either ATRX loss or ALT were later diagnosed with a pulmonary smooth muscle tumor. Pulmonary tumors displayed molecular alterations found in the corresponding uterine tumors, which indicated metastasis to the lungs. All conventional ULs displayed normal ATRX, DAXX, and telomeres. **CONCLUSIONS:** These results highlight the differences between conventional and histopathologically atypical ULs and indicate that some UL subtype tumors may harbor long-term malignant potential. ***Cancer* 2018;124:4650-4656.** © *2018 American Cancer Society*.

KEYWORDS: alpha-thalassemia/mental retardation syndrome X-linked (ATRX), death domain–associated protein (DAXX), alternative lengthening of telomeres (ALT), benign metastasizing leiomyoma, uterine leiomyoma, uterine leiomyosarcoma.

INTRODUCTION

Uterine leiomyomas (ULs) are the most common gynecologic neoplasms and affect approximately 75% of women by the age of 50 years.[1] Despite their benign nature, ULs can disturb the uterine function and cause several health complications such as abnormal bleeding, pelvic discomfort, and reproductive dysfunction. ULs are the leading cause of hysterectomy and cause a massive economic burden for health care systems.[1] High-throughput sequencing has revealed that approximately 70% of conventional ULs harbor mutations in mediator complex subunit 12 (*MED12*).[2] Other recurrent alterations include high-mobility group AT-hook 2 (HMGA2) overexpression (10%-20%) and fumarate hydratase (*FH*) inactivation (1%-2%), and together, these 3 driver aberrations account for 80% to 90% of the tumors.[3]

In addition to conventional ULs, there are several histopathological UL subtypes (herein called variants), such as cellular and mitotically active tumors and ULs with bizarre nuclei.[4] These variant tumors are also considered clinically benign and are treated accordingly, although some of their histopathological traits mimic malignancy. *MED12*, HMGA2, and FH alterations can be found in UL variants, but their combined contribution is considerably lower than that in conventional ULs.[5,6]

Uterine leiomyosarcoma (ULMS) is considered a malignant counterpart of UL. It is a rare but highly aggressive cancer with poor 5-year survival.[7] The early diagnosis and preoperative differentiation of ULMS from UL are often challenging because of highly overlapping symptoms, and this raises the clinical concern that some ULMSs are treated and operated on as benign ULs. Currently, a postoperative histopathological examination is the only reliable method

Corresponding author: Pia M. Vahteristo, PhD, Department of Medical and Clinical Genetics, Biomedicum Helsinki, P.O. Box 63 (Haartmaninkatu 8), FIN-00014 University of Helsinki, Helsinki, Finland; pia.vahteristo@helsinki.fi

[1]Genome-Scale Biology Research Program, Research Programs Unit, University of Helsinki, Helsinki, Finland; [2]Department of Medical and Clinical Genetics, Medicum, University of Helsinki, Helsinki, Finland; [3]Department of Pathology, Laboratory of Helsinki University Central Hospital, Helsinki University Central Hospital and Medicum, University of Helsinki, Helsinki, Finland.

We thank Assistant Professor L. Kauppi for consulting on the telomere length classification and A. Ruokolainen and L. Honkala for their technical assistance. We acknowledge Biomedicum Imaging Unit (Helsinki, Finland) for providing the facilities for microscopy imaging and digitalization and Genome Biology Unit (Helsinki, Finland) for tissue microarray image scanning and digitalization.

DOI: 10.1002/cncr.31754, **Received:** June 18 2018; **Revised:** July 27 2018; **Accepted**: August 6 2018, **Published online** November 13, 2018 in Wiley Online Library (wileyonlinelibrary.com)

to distinguish ULMS from UL. Despite recent advances, the pathogenesis of ULMS remains poorly understood. Recently, we and others have shown that the expression of alpha-thalassemia/mental retardation syndrome X-linked (ATRX) is lost in 39% to 52% and the expression of death domain–associated protein (DAXX) is lost in 0% to 2% of ULMSs.[8,9] A loss of ATRX/DAXX has been associated with the alternative lengthening of telomeres (ALT) phenotype, which uses homologous recombination for telomere length maintenance instead of activation of the telomerase enzyme.[10] ALT is used especially by brain tumors and soft-tissue sarcomas,[11] and it has also been observed in the majority of ULMSs.[8,9]

Frequent loss of ATRX/DAXX expression and ALT in ULMSs prompted us to study whether these aberrations could be seen in variant or conventional ULs. The presence of such alterations could indicate that some ULs harbor malignant potential and represent ULMS precursor lesions.

MATERIALS AND METHODS

Two hundred six formalin-fixed, paraffin-embedded tumor samples representing several histopathological UL variants (n = 142) and conventional ULs (n = 64) were derived from the Department of Pathology of Helsinki University Hospital (Helsinki, Finland). The histopathology was reexamined via the corresponding hematoxylin-eosin–stained slides by a pathologist specialized in gynecologic pathology (R.C.B. or A.M.P.). The variant UL samples include 35 cellular ULs, 45 highly cellular ULs, 31 mitotically active ULs (including 15 tumors also displaying cellularity [n = 7] or high cellularity [n = 8]), and 31 ULs with bizarre nuclei. In addition to uterine tumors, 3 pulmonary smooth muscle tumor samples from patients who were earlier treated for a UL variant were obtained, and the diagnoses were reexamined by a pathologist (R.C.B.) via the corresponding hematoxylin-eosin slides. Two pulmonary tumor samples with high cellularity were obtained from the removed tumors, whereas the sample for the pulmonary tumor with bizarre nuclei was obtained via core-needle biopsy. The study was approved by the appropriate ethics committee of the Hospital District of Helsinki and Uusimaa, Helsinki, Finland (88/13/03/03/2015), and the National Supervisory Authority for Welfare and Health (8522/06.01.03.01/2015), and it is in accordance with the Declaration of Helsinki.

The presence of UL driver mutations was previously analyzed for all conventional tumors and for a subset of variant tumors.[6] Similar analyses were now performed for the remaining samples. In brief, *MED12* exons 1 and 2 were analyzed by direct sequencing, and HMGA2 and FH expression was analyzed by immunohistochemistry. For immunohistochemical analyses, tissue microarrays were constructed as previously described.[6] HMGA2 expression was analyzed with an anti-HMGA2 antibody (1:2000; 59170AP; Biocheck, Inc, Foster City, California), and FH inactivation was assessed with an anti-FH antibody (1:1000; clone J-13, sc-100743; Santa Cruz Biotechnology, Inc, Dallas, Texas). ATRX and DAXX immunolabeling was performed with anti-ATRX (1:500; HPA001906; Sigma-Aldrich, St. Louis, Missouri) and anti-DAXX antibodies (1:500; HPA008736; Sigma-Aldrich) as previously described.[8] Whole sections were analyzed from samples that showed aberrant expression or failed on tissue microarrays. All staining was analyzed and scored by a pathologist (R.C.B.) with a 3-grade scale: strong expression (++), normal/weak expression (+), or no expression (–). Retained staining in endothelial or inflammatory cells was used as an internal positive control.

Telomere-specific fluorescence in situ hybridization was applied to determine the presence of ALT. A cyanine 3–labeled peptide nucleic acid probe (F1006-5; Panagene, Daejeon, South Korea) was used as previously described.[8] The imaging and digitalization of tissue microarray sections were performed with a Pannoramic P250 Flash II digital slide scanner (3DHISTECH, Budapest, Hungary) equipped with a Zeiss Plan-Apochromat 40×/NA 0.95 objective and a pco.edge 4.2 complementary metal-oxide semiconductor camera (CMOS) along with the DAPI shift-free (E) filter set (DAPI-1160B-000) and the 43 HE Cy 3 shift-free (E) filter set (489043-9901-000). Pannoramic Viewer (version 1.15.3; 3DHISTEC) was used to annotate samples. The whole tissue sections were imaged with a Zeiss Axio Imager.Z2 upright epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Hamamatsu Orca Flash 4.0 LT 4-megapixel monochrome scientific CMOS digital camera (Hamamatsu Photonics, Hamamatsu, Japan) and Zeiss Zen 2 Pro software. ALT was scored independently by 2 authors (T.V.A. and N.M.M./P.v.N.), and 300 cells were calculated from each sample. Samples displaying big, heterogeneous, and abnormally bright intranuclear DNA foci in ≥5% of the cells were considered positive. Whole sections were analyzed from samples considered positive or showing aberrant or unclear telomeres on tissue microarrays.

RESULTS

Here we analyzed ATRX and DAXX expression and the presence of ALT in 142 variant ULs and 64

TABLE 1. ATRX and DAXX Expression and ALT in Uterine Leiomyomas

Histopathology	No.	*MED12* Mutation	HMGA2 Overexpression	ATRX Loss	DAXX Loss	ALT+	ATRX Loss and ALT+	ATRX Loss/DAXX Loss/ALT+
Conventional	64	37 (58)	16 (25)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Histopathological subtypes	142	21 (15)	19 (13)	6 (4.2)	1 (0.7)	2 (1.4)	2 (1.4)	9 (6.3)
Highly cellular	45	1 (2.2)	7 (16)	1 (2.2)	0 (0)	0 (0)	0 (0)	1 (2.2)
Cellular	35	6 (17)	10 (29)	0 (0)	1 (2.9)	0 (0)	0 (0)	1 (2.9)
Bizarre nuclei	31	4 (13)	0 (0)	4 (13)	0 (0)	1 (3.2)	2 (6.5)	5 (16)
Mitotically active	16	7 (44)	2 (13)	1 (6.3)	0 (0)	0 (0)	0 (0)	1 (6.3)
(Highly) cellular and mitotically active	15	3 (20)	0 (0)	0 (0)	0 (0)	1 (6.7)	0 (0)	1 (6.7)

Abbreviations: ALT, alternative lengthening of telomeres; ATRX, alpha-thalassemia/mental retardation syndrome X-linked; DAXX, death domain–associated protein; HMGA2, high-mobility group AT-hook 2; MED12, mediator complex subunit 12.
Percentages are presented in parentheses.

conventional ULs. Aberrations were detected in 9 variant ULs (9 of 142 [6.3%]; Table 1 and Fig. 1A): ATRX expression was lost in 6 tumors (6 of 142 [4.2%]), 1 tumor showed DAXX loss (1 of 142 [0.7%]), and 4 tumors displayed ALT (4 of 142 [2.8%]). The majority of the tumors harbored only 1 aberration (7 of 9 [77.8%]), whereas 2 tumors showed both ATRX loss and ALT. We also identified 2 ALT-positive tumors, which expressed normal levels of both ATRX and DAXX; this indicates that there are other mechanisms that underlie the alternating telomere length in these tumors. Aberrations were found in all histopathological UL variant subgroups studied. All conventional ULs had normal levels of ATRX and DAXX expression, and none displayed ALT.

The great majority of conventional ULs harbor mutations in *MED12* or overexpress HMGA2.[3] Tumors with either one of these driver mutations display relatively few other mutations.[12] Here, 53 of 64 conventional tumors (83%) displayed either a *MED12* mutation or HMGA2 overexpression. In contrast, *MED12* mutations or HMGA2 overexpression were identified in only 40 of 142 variant ULs (28%). All tumors with ATRX/DAXX loss or ALT were wild-type for *MED12* and HMGA2 (Table 1).

We further examined the medical histories of 9 patients whose tumors displayed ATRX/DAXX loss and/or ALT. Two patients were later diagnosed with pulmonary smooth muscle tumors (Table 2 and Fig. 1B). One of these 2 patients had a pulmonary leiomyoma 12 years after hysterectomy. The uterine and pulmonary leiomyomas displayed highly similar histologies, including bizarre nuclei. The uterine tumor was ALT-positive, and the pulmonary tumor showed both ATRX loss and ALT. The other patient was diagnosed with 2 pulmonary leiomyosarcomas, the first 8 years and the second 11 years after hysterectomy. The uterine tumor showed highly cellular histopathology, ATRX loss, and FH loss, whereas both pulmonary sarcomas displayed ATRX loss, ALT, and FH loss. These findings suggest dissemination of the uterine tumors to the lungs.

DISCUSSION

ATRX is a chromatin remodeling protein that interacts with DAXX and plays a role in telomere integrity.[10] Dysfunctional ATRX and DAXX have been associated with the ALT phenotype, and a strong correlation between the loss of ATRX or DAXX expression and ALT has been observed in several malignancies, including ULMS.[8-11] Currently, the role of ATRX/DAXX in ALT is not well understood. ALT-positive ULMSs with no detectable loss of ATRX or DAXX have been reported, and this indicates that other mechanisms contribute to telomere length in these tumors.[9] Here, 6.3% of UL variants (9 of 142) had a loss of ATRX or DAXX and/or ALT, whereas all conventional ULs displayed normal expression and telomeres. These aberrations were found in variant tumors that were negative for *MED12* and HMGA2 aberrations, and this further emphasizes the differences between the molecular characteristics and pathogenesis mechanisms of conventional and variant ULs. Two of the 9 samples displayed both a loss of ATRX expression and ALT, whereas 7 tumors displayed only 1 aberration, suggesting that other mechanisms regulate the manifestation of ALT hallmarks in these tumors.

The leiomyoma subtype with the most frequent ATRX, DAXX, and/or ALT aberrations was UL with bizarre nuclei because 5 of the 31 tumors (16%) displayed alterations. These tumors are characterized by irregularly shaped nuclei or several hyperchromatic nuclei and eosinophilic cytoplasm.[4] Accumulating evidence suggests that their genetic and clinical characteristics share traits with those of ULMS, and this in part separates this group from other ULs. The most common molecular defect in these tumors is biallelic *FH* inactivation, which has been identified in more than 30% of tumors and is much more common in this UL subtype than other ULs.[6,13] *MED12*

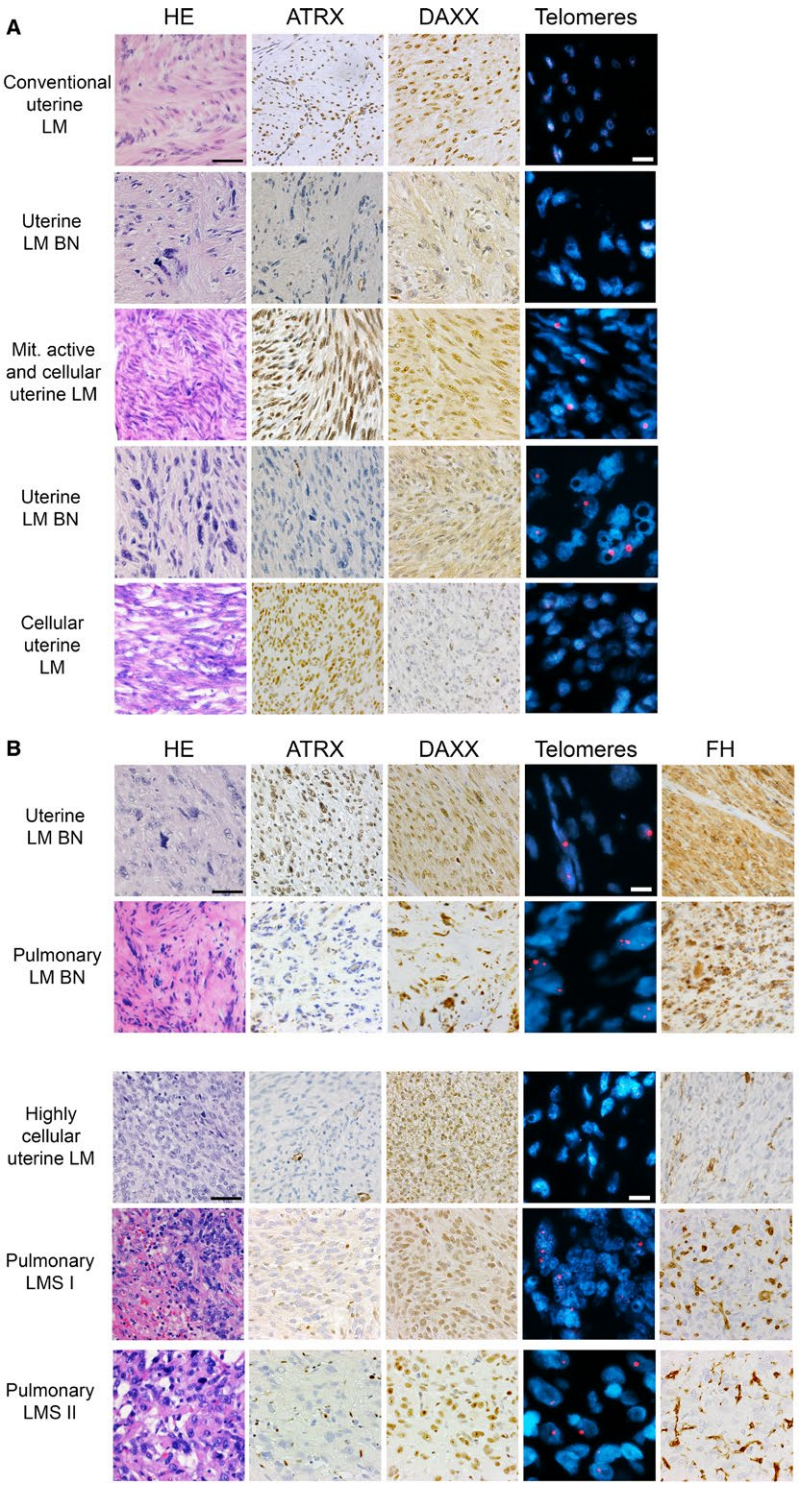

Figure 1. (A) Representative images of histopathology, ATRX/DAXX expression, and telomere lengths in uterine LMs: conventional LM with normal ATRX and DAXX expression and telomeres; LM BN with ATRX loss and normal DAXX expression and telomeres; mitotically active and cellular LM with normal ATRX and DAXX expression and ALT; LM BN with ATRX loss, normal DAXX expression, and ALT; and cellular LM with normal ATRX expression, DAXX loss, and normal telomeres. (B) ATRX/DAXX expression and telomere lengths in uterine LMs and pulmonary smooth muscle tumors from 2 patients. The uterine-pulmonary LM BN pair included a uterine tumor with normal ATRX, DAXX, and FH expression and ALT and a pulmonary tumor with a loss of ATRX, ALT, and normal expression of DAXX and FH. The highly cellular uterine LM–pulmonary LMS pairs included a uterine tumor with a loss of ATRX and FH expression and normal DAXX expression and telomeres and pulmonary LMS I and pulmonary LMS II (operated 8 and 11 years after hysterectomy, respectively) with a loss of ATRX and FH, normal DAXX expression, and ALT. ALT indicates alternative lengthening of telomeres; ATRX, alpha-thalassemia/mental retardation syndrome X-linked; DAXX, death domain–associated protein; FH, fumarate hydratase; HE, hematoxylin-eosin; LM, leiomyoma; LM BN, leiomyoma with bizarre nuclei; LMS, leiomyosarcoma. Scale bar 50 μm for HE and IHC images, and 10 μm for telomere-specific FISH images.

mutations, in turn, are significantly less frequent in these tumors than in conventional ULs, and the mutation frequency resembles that observed in ULMSs.[5,8,14] Also, *TP53* mutations, which are some of the most common alterations in ULMSs,[8,14,15] have been identified in ULs with bizarre nuclei.[14] These tumors reoccur more often than other UL subtypes.[14,16] Although ULMSs are considered to develop as de novo tumors, microscopically visible colocalization with ULs with bizarre nuclei has been reported together with shared genetic changes.[17] These findings support the hypothesis that some ULs with bizarre nuclei may harbor malignant potential and progress into ULMSs.

Cellular and especially highly cellular ULs have substantially increased cellularity in comparison with nearby myometrium, and their features resemble, in part, those associated with endometrial stromal tumors.[4] Genetic findings indicate that the molecular pathogenesis of highly cellular ULs mostly differs from that of conventional ULs; these tumors harbor the least amount of known UL driver aberrations, with nearly 80% of the tumors having no identified underlying molecular defect.[6] Here, 1 highly cellular tumor showed a loss of ATRX expression (1 of 45 [2.2%]), and 1 cellular tumor showed a loss of DAXX expression (1 of 35 [2.9%]). Although cellular and highly cellular ULs share some features with ULMSs, the key molecular defects leading to the development of these tumors remain to be determined.

Mitotically active ULs are defined as having more than 10 mitoses per 10 high-power fields.[4] In contrast to cellular ULs and ULs with bizarre nuclei, mitotically active ULs have a relatively high *MED12* mutation frequency, and they have not been shown to harbor alterations typically associated with malignant tumors.[6,14] In this study, 1 mitotically active tumor showed a loss of ATRX expression (1 of 16 [6.3%]), and 1 tumor with both increased cellularity and mitotic activity displayed ALT (1 of 15 [6.7%]). Thus, some tumors of this UL subtype also have mutations associated with malignancy.

When the medical history was scrutinized for the 9 patients whose tumors showed ATRX/DAXX loss and/or ALT, 2 patients were found to have been diagnosed with pulmonary smooth muscle tumors years after UL removal. Pulmonary and uterine tumors displayed similar molecular alterations, and this suggested possible extrauterine dissemination of ULs and thus a condition known as benign metastasizing leiomyoma (BML) in these patients. BML is a rare disease in which women with a previous history of UL develop smooth muscle tumors outside the uterus. The lungs are the predominant site of metastasis, and the average time of the pulmonary tumor diagnosis is 8.8 years after UL removal.[18] The clinical course of BML is typically indolent, although rare malignant transformation has been reported.[19,20] The exact mechanism of BML pathogenesis remains unresolved, but pulmonary metastasis via hematogenous circulation has been suggested. Identical genetic aberrations and elongated telomeres have been identified in UL–pulmonary tumor pairs, and this suggests the clonal origin of these tumors.[21-23] One of the patients identified here had an FH-deficient UL, and the pulmonary tumors also displayed FH loss. FH loss is a known but rare driver event in UL and leads to severe metabolic stress due to deficient tricarboxylic acid cycle function. One BML patient with a germline *FH* mutation has been reported, but the FH status of the tumors is not known.[24] Further studies with additional samples are warranted to verify the possible contributions of ATRX, ALT, and/or FH to BML pathogenesis.

In conclusion, we here show that a subset of UL variants display ATRX or DAXX loss and/or ALT. These features have been associated with several malignancies, especially brain tumors and sarcomas.[8-11] Aberrations were identified in each UL subtype but in none of the conventional tumors. Two patients with ATRX loss or ALT were later diagnosed with a pulmonary smooth muscle tumor, and this suggested extrauterine dissemination. Together with the previous data, these results

TABLE 2. Nine Patients Whose Uterine Leiomyomas Displayed a Loss of ATRX/DAXX and/or ALT

Patient ID	Age at Hysterectomy (Year of Operation)	Features of Uterine Tumor								Pulmonary Tumor (Time From Hysterectomy)	Alterations in Pulmonary Tumor(s)
		Histopathology	Driver Event	Mitotic Index	Nuclear Atypia	Cellularity	ATRX Expression	DAXX Expression	ALT		
D8[a]	54 (2003)	LM with BN	WT	<5	1-3	+	Positive	Positive	Positive	LM with BN (12 y)	ATRX-negative, ALT-positive
D14[a]	58 (2007)	LM with BN	WT	<5	3	+	Negative	Positive	Positive	—	—
1322	51 (2016)	LM with BN	WT	<5	3	+	Negative	Positive	Positive	—	—
C21[a]	45 (1996)	LM with BN	FH	<5	1-3	–	Negative	Positive	Negative	—	—
1338	45 (2013)	LM with BN	WT	<5	0-2	–	Negative	Positive	Negative	—	—
D31[a]	63 (2004)	Highly cellular	FH	<5	1	++	Negative	Positive	Negative	LMS (8 and 11 y)	ATRX-negative, ALT-positive, FH-negative
D7[a]	41 (2004)	Mitotically active and cellular	WT	20	1	+	Positive	Positive	Positive	—	—
1326	46 (2012)	Mitotically active	WT	5-10	0	–	Negative	Positive	Negative	—	—
1364	43 (2016)	Cellular	WT	<5	1	+	Positive	Negative	Negative	—	—

Abbreviations: ALT, alternative lengthening of telomeres; ATRX, alpha-thalassemia/mental retardation syndrome X-linked; BN, bizarre nuclei; DAXX, death domain–associated protein; FH, fumarate hydratase; HMGA2, high-mobility group AT-hook 2; LM, leiomyoma; LMS, leiomyosarcoma; MED12, mediator complex subunit 12; WT, wild type for the known leiomyoma driver mutations (*MED12*, *HMGA2*, and *FH*).
[a]Patient numbers are taken from Makinen et al.[6]

highlight the differences in the molecular pathogenesis of conventional ULs and UL variants. The findings also raise concern about the long-term malignant potential of some UL variants, which are currently treated and operated on as benign tumors. Additional data are required to determine the potential role of ATRX and/or ALT in malignant transformation and to evaluate their feasibility in diagnostics. The identification of tumors with malignant potential could affect treatment procedures and eventually prevent malignant transformation.

FUNDING SUPPORT

This work was supported by the Academy of Finland (260370 and 307773 to Pia M. Vahteristo), the Sigrid Jusélius Foundation, and the Cancer Society of Finland. Netta M. Mäkinen was supported by the Academy of Finland, and Pernilla von Nandelstadh was supported by the Waldemar von Frenkells Foundation, the Medical Society for Life and Health, and the K. Albin Johanssons Foundation.

CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

AUTHOR CONTRIBUTIONS

Terhi V. Ahvenainen: Formal analysis, investigation, and writing–original draft. **Netta M. Mäkinen:** Formal analysis, investigation, and writing–original draft. **Pernilla von Nandelstadh:** Formal analysis, investigation, and writing–original draft. **Maija E. A. Vahteristo:** Investigation. **Annukka M. Pasanen:** Resources. **Ralf C. Bützow:** Formal analysis and resources. **Pia M. Vahteristo:** Conceptualization, writing–original draft, supervision, project administration management, and funding acquisition.

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